RECEIVED
CENTRAL FAX CENTER
DEC 06 2011

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S)	: BOWEN, Philip J. et al
SERIAL NO.	: 10/502,080
FILED	: October 8, 2004
FOR	: SOLENOPSIN A, B AND ANALOGS AS NOVEL ANGIOGENESIS INHIBITORS
GROUP ART UNIT	: 1628
Examiner	: Paul E. Zarek

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

FURTHER DECLARATION OF DR. JACK L. ARBISER

I, Jack L. Arbiser declare as follows:

1. I am a co-inventor of the subject matter of the above-referenced patent application.

2. In 1983, I received a B.S. degree in Chemistry from Emory University, Atlanta, Georgia.

3. In 1991, I received a Ph.D. degree in Genetics and a MD degree in Medicine from Harvard Medical School, Boston, Massachusetts.

5. From 1994-1998, I participated in the Howard Hughes Postdoctoral Fellowship, Laboratory of Judah Folkman, M.D., Harvard Medical School, Boston Massachusetts.

6. Since 1991, I have studied the mechanisms of how oncogenes and tumor suppressor genes regulate angiogenesis and tumorigenesis. This work has resulted in the

PAGE 16/45 * RCVD AT 12/6/2011 9:37:37 AM [Eastern Standard Time] * SVR:W-PTOFAX-001/26 * DNIS:2738300 * CSID:12038706002 * DURATION (mm-ss):09-35

discovery of small molecule inhibitors of tumor growth, and a novel method of predicting tumor signaling based upon loss of tumor suppressor genes p53 and p19ink4a. I have substantial scientific and medical expertise in cancer and clinical oncology.

7. I am presently a Professor of Dermatology, Emory University School of Medicine, Atlanta, Georgia. I have held this position since September, 2009.

8. I am also presently an Attending Physician, Atlanta Veterans Administration Medical Center, Atlanta Georgia. I have held this position since 2001.

9. In 2008, I was the Chief of Service of Dermatology and the Chief of Dermatology, Atlanta Veterans Administration Medical Center, Atlanta Georgia.

10. In 2007, I was the Director of Research, Department of Dermatology, Emory University School of Medicine, Atlanta, Georgia.

11. From 2004 to 2009, I was an Associate Professor in the Department of Dermatology, Emory University School of Medicine, Atlanta, Georgia.

12. From 1998 to 2004, I was an Assistant Professor in the Department of Dermatology, Emory University School of Medicine, Atlanta, Georgia.

13. In 1998, I was an Attending Physician, Emory University School of Medicine, Atlanta, Georgia

14. From 1995 to 1998, I was an Instructor in the Department of Dermatology, Harvard Medical School, Boston, Massachusetts.

15. From 1992 to 1994, I was a Resident in Dermatology, Massachusetts General

Hospital, Boston, Massachusetts.

16. From 1991 to 1992, I was an Intern in Internal Medicine, Beth Israel Hospital, Boston, Massachusetts.

17. From 1985 to 1991, I was in the Medical Scientist Training Program, Department of Genetics, Harvard Medical School, Boston, Massachusetts.

18. In 1984, I was a Research Assistant, Department of Rheumatology, Massachusetts General Hospital, Boston, Massachusetts.

19. In 1983, I was a Research Assistant, Department of Pediatrics, Emory University, Atlanta, Georgia.

20. In 1979, I was an Undergraduate Research Assistant, Department of Chemistry, Emory University, Atlanta, Georgia.

21. I have received numerous awards and honors for my scientific work including receiving the Albert E. Levy Scientific Research Award for Senior Investigator in 2007, and receiving the Emory School of Medicine Dean's Clinical Scholar award from 2000-2003 and 2004-2006 among other awards and honors.

22. I am a member of the Emory Medical Student Research Committee (2001-present) and the VA Research and Development Committee (2007-present). I am also a member of the Dermatology Foundation Medical and Scientific Committee External Advisory Board, University of Arizona Cancer Center, the Sturge-Weber Foundation Scientific Advisory Board (2001-present) and the American Academy of Dermatology-NAID Liaison (1998-present). I was an Organizer for the 48th Montagna Annual Symposium on the Biology of Skin, Snowmass Colorado (1999). I have been a Membership Chair of the Society for Investigative Dermatology

PAGE 18/45 * RCVD AT 12/6/2011 9:37:37 AM [Eastern Standard Time] * SVR:W-PTOFAX-001/26 * DNIS:2738300 * CSID:12038706002 * DURATION (mm-ss):09-35

(2001-2002) and a Resident/Fellow Representative for the Society of Investigative Dermatology (1995-1997).

23. I have been a member of the following societies: the American Association for Cancer Research, The Society for Investigative Dermatology, the American Academy of Dermatology, the Tuberous Sclerosis Alliance, the Dermatology Foundation and the Sturge-Weber Foundation.

24. I am on the Editorial Boards for Pigment Cell Research (2007-present) and Journal of Investigative Dermatology (2001-present). I have been on the Editorial Boards for Journal of the Cutaneous Medicine and Surgery (2002-2005) and Journal of the American Academy of Dermatology (2001-2004). I was also a Guest Editor for Seminars in Cancer Biology, Karolinska Institute.

25. I have published over 200 scientific papers and I have published extensively in the scientific area of cancer research, including the mechanism by which cancer occurs, including the role of angiogenesis in cancer pathogenesis, including tumorigenesis.

26. The above paragraphs clearly establish a foundation for my medical and scientific expertise in cancer, including experimental cancer and clinical oncology, in providing the instant declaration.

27. I am familiar with United States patent application serial number 10/502,080, of which I am a co-inventor. I understand that the presently pending claims are directed to a method of treating cancer or a tumor in a patient comprising administering to a patient in need an effective amount of a composition which comprises a compound as otherwise set forth in the presently pending claims, namely claims 40, 50-56 and 66. Essentially, the presently pending claims are directed to the discovery that compositions which contain effective amounts of a compound as claimed are effective to treat a number of cancers and tumors. This is based upon

PAGE 19/45 * RCVD AT 12/6/2011 9:37:37 AM [Eastern Standard Time] * SVR:W-PTOFAX-001/26 * DNIS:2738300 * CSID:12038706002 * DURATION (mm-ss):09-35

the fact that the compounds which are set forth in presently pending claims 40, 50-56 and 66 inhibit cancer and/or tumor growth by a mechanism which inhibits angiogenesis in the cancer/tumor tissue. By inhibiting angiogenesis, the presently claimed methods provide a generic approach to the treatment of any number of cancers and tumors as set forth in presently pending claims 40, 50-56 and 66.

28. It is my opinion that the presently claimed methods of treating tumors and/or cancer are useful and are expected to work as described and claimed, given that angiogenesis is an important generic mechanism by the way tumors and/or cancer grow and elaborate, and the presently claimed methods set forth in pending claims 40, 50-56 and 66 are directed to methods which utilize the inhibition of angiogenesis as a general mechanism to treat tumor and/or cancer. The compound which is claimed for use in the present method, solenopsin A, is an excellent anti-angiogenesis compound which exhibits broad activity against a large number of cancers, reflective of that mechanism as an angiogenesis inhibitor.

29. Angiogenesis comprises the development of a new vasculature for a tissue with increased metabolic demand. In adult life, the new tissue is likely to be a tumor or cancer, either benign or malignant, or an inflammatory process, such as psoriasis, inflammatory bowel disease, arthritis, asthma, multiple sclerosis, type II diabetes, lupus, and other diseases. The major sources of the blood vessel cells (endothelial cells) that are required for this process are recruitment of blood vessel cells from local pre-existing capillaries, or recruitment of cells from bone marrow that can turn into endothelial cells. Both processes contribute to the vascularization of a tumor or an inflammatory process. One of the commonalities of both inflammatory and tumor derived (neoplastic processes) is that they elaborate factors that recruit endothelial cells. The major factors for these processes include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), inflammatory cytokines (such as interleukin-8, and other factors), which stimulate the migration and proliferation of endothelial cells. The laboratory of Judah Folkman, MD, proved the absolute requirement of angiogenesis for the growth of malignant tumors. Based upon Dr. Folkman's pioneering work, angiogenesis

PAGE 20/45 * RCVD AT 12/6/2011 9:37:37 AM [Eastern Standard Time] * SVR:W-PTOFAX-001/26 * DNIS:2738300 * CSID:12038706002 * DURATION (mm-ss):09-35

inhibitors have been developed for the treatment of human diseases, in particular the treatment of tumors and cancer.

26. Two strategies have been developed for the assessment of angiogenesis inhibitors. The first is direct inhibition, in which the activity of growth factors on the receptors is directly antagonized. The second is indirect inhibition, in which the ability of tumors to produce growth factors is inhibited. The phosphoinositol-3 kinase pathway, which is activated in virtually all tumors, is implicated in both direct and indirect angiogenesis inhibition.

27. Direct antiangiogenesis inhibition is now in clinical use for the treatment of cancer. The most prominent example is that of avastin (bevacizumab) that directly blocks the activity of VEGF on endothelial cells. Avastin is commonly used for the treatment of kidney and colon cancer, and more recently in brain cancer (glioblastoma). While avastin has been shown to be of clinical benefit, it is not curative and has well known side effects, such as hypertension and bleeding. In addition, the tumor hypoxia that is induced by avastin is thought to cause adaptation in the tumor, such as increased local invasion and elaboration of more growth factors in order to relieve the avastin-induced tumor hypoxia. Other strategies are being developed, but it is likely that any strategy that increased tumor hypoxia by itself will ultimately not cure a tumor.

28. Indirect inhibition of angiogenesis is an attractive strategy that has not been sufficiently explored. My studies in the Folkman lab, published in the Proceedings of the National Academy, were the first to demonstrate that blockade of phosphoinositol-3 kinase was able to inhibit the growth of a tumor *in vivo*. Blockade of phosphoinositol-3 kinase is an attractive strategy for several reasons. First, it blocks the production of growth factors by the tumor. Second, it causes increased apoptosis (programmed cell death) in tumors themselves. Finally, it is believed to prevent the metabolic adaptations in tumors caused by antiangiogenic therapies. Since phosphoinositol-3 kinase is such an important target, we regard it as a major focus to inhibit this pathway and to treat tumors and cancer. We discovered that solenopsin, a

PAGE 21/45 * RCVD AT 12/6/2011 9:37:37 AM [Eastern Standard Time] * SVR:W-PTOFAX-001/26 * DNIS:2738300 * CSID:12038706002 * DURATION (mm-ss):09-35

naturally occurring alkaloid in the venom of the fire ant (Solenopsis invicta), is a potent inhibitor of this enzyme. In addition, we have shown that this compound claimed in the present invention is a potent inhibitor of angiogenesis in the zebrafish model and that solenopsin is stable and may be used as a pharmaceutical agent. See the previously submitted references, Arbiser, et al, *Blood*, 15 January 2007, Volume 109, Number 2, pages 560-565, which teaches the inhibition of angiogenesis by solenopsin consistent with its use as an anticancer agent, and Park, et al., *Journal of Infectious Diseases*, 15 October 2008, 198, 1198-201, which teaches that solenopsin is stable and may be used as a small molecule pharmaceutical agent as set forth in the present invention. The inhibitory activity, small molecular size and stability of solenopsin, which make it amenable to topical, systemic and oral administration, make it an attractive molecule for the treatment of tumors and cancer. It thus represents close to an ideal compound for providing generic therapy against a variety of cancerous tissue.

29. By way of cellular experimental evidence, recently the following experiments on a benign tumor cell line (FP52-SV) and malignant sarcoma cell (tsc2ang1) were conducted. In these experiments, the anti-proliferative/anticancer activity of solenopsin A (a compound claimed in the present application) were tested in the two cell lines. More specifically, 10,000 cells from benign tumors (FP52-SV40) or malignant sarcoma cells (tsc2ang1) were plated in 24 well plates. 24 hours after plating, the cells were treated with solenopsin in concentrations ranging from 0-20 micromolar or vehicle control. A number of other compounds were also tested. 24 hours after solenopsin treatment, the cells were trypsinized and counted with a cell culture.

30. The results of the experiments which are described in paragraph 29, above are shown in the attached Exhibits 1 and 2. In the first assay, the proliferation assay performed on the benign tumor cell line FP52 SV40, the anti-proliferative effect of solenopsin on the cells is clearly evidenced (see attached Exhibit 1). Noted is the fact that a concentration of solenopsin at 10 micromolar provided significant inhibition of cell proliferation (approximately 80%). The graph also evidences that anti-proliferative activity of solenopsin was synergistic with

PAGE 22/45 * RCVD AT 12/6/2011 9:37:37 AM [Eastern Standard Time] * SVR:W-PTOFAX-001/26 * DNIS:2738300 * CSID:12038706002 * DURATION (mm-ss):09-35

imipramine blue where both compounds were used at a concentration of 1 micromolar. In the second experiment, the effect of solenopsin at varying concentrations was tested against malignant sarcoma TSC2ang1 cells (Exhibit 2). In this experiment, solenopsin exhibited excellent antiproliferative/anticancer activity against the sarcoma cell line with a concentration of 10 micromolar solenopsin being particularly effective, with concentration of solenopsin of 15 and 20 micromolar being slightly less effective than the lower concentration of 10 micromolar. In a third experiment, solenopsin was tested against a malignant melanoma cell line A375 (Exhibit 3). In this experiment, solenopsin exhibited excellent antiproliferative/anticancer activity against the melanoma cell line at a concentration of 10 micromolar with significant anticancer activity at a concentration of 10 micromolar. In all three experiments, the results of which are presented in attached Exhibits 1, 2 and 3, solenopsin showed substantial antiproliferative/anticancer activity in cell-based assays consistent with its use as a generic anticancer agent as claimed in the present invention.

31. Recently, solenopsin A was tested by the National Cancer Institute (NCI) against a number of cancer cell lines. This *in vitro* testing was conducted in 60 human tumor cell lines in the following cancers: breast, central nervous system, colon, leukemia, melanoma, non-small cell lung, ovarian, prostate and renal. Pursuant to NSC guidelines, each drug (in this case, NSC 166588 (Solenopsin A) is exposed to 60 humor tumor cell lines of the various cancer cited above at five different doses for 48 hours. The results of the *in vitro* testing in the 60 cell lines is presented in attached Exhibit 4. The NCI results evidenced that in all of the tumor cell lines tested, solenopsin exhibited substantial anti-cancer activity at 1 to 100 micromolar concentrations, depending on the cell line, consistent with its use as an anti-cancer agent. All cell lines were impacted by the Solenopsin treatment, evidencing that Solenopsin exhibited anti-cancer activity against every cell line in the 60 cell-line panel.

32. In addition to the experimental data which is presented in attached Exhibits 1-4, which clearly evidences that solenopsin exhibits generic anticancer activity against a number of different cancers and a large number of cell lines, the mechanism of action of

PAGE 23/45 * RCVD AT 12/6/2011 9:37:37 AM [Eastern Standard Time] * SVR:W-PTOFAX-001/26 * DNIS:2738300 * CSID:12038706002 * DURATION (mm-ss):09-35

solenopsin and its anticancer activity is completely consistent with its use in a number of additional cancers. In particular, a review of the literature evidences that the inhibition of phosphatidyl inositol 3-kinase as part of an anti-angiogenesis mechanism is relevant to cancer treatment in a broad range of cancers, including skin cancer, colorectal cancer, head and neck cancer, breast cancer, including metaplastic breast cancer, lung cancer, pancreatic cancer and skin cancers, including basal cell carcinoma, squamous cell carcinoma and melanoma. In support of the generic utility of the present invention as a treatment for numerous cancers, enclosed herewith are a number of peer reviewed publications in the scientific literature which evidences that inhibition of phosphatidyl inositol 3-kinase (the inhibition of the pathway through which solenopsin exhibits its generic anti-cancer effect) is consistent with cancer growth inhibition and apoptosis of cancer cells. This mechanism is shown to be important for the treatment of a number of cancers including skin cancers (see Anto, et al., *The Journal of Biological Chemistry*, 278, 28, pp. 25490-25498. August, 2003, copy enclosed), colorectal cancer (see Baba, et al., *Cancer,* April 1, 2011, pp. 1399-1408, copy enclosed), head and neck cancer (see Bian, et al., *Cancer Res.,* 2009, July 15; 69(14), pp. 5918-5926, copy enclosed), breast cancer (see Capodanno, et al., *Human Pathology,* 2009, 40, 1408-1417 and Hennessy, et al., *Cancer Res.,* 2009, May 15, 69(10) pp. 4116-4124, copies enclosed), lung cancer (see Capuzzo, et al., *Journal of the National Cancer Institute*, 96, 15, August 4, 2004, pp. 1133-1141, copy enclosed), pancreatic cancer (Chen, et al., *Pathol. Oncol. Res.,* 2011, 17:257 pp. 257-261, copy enclosed), skin cancers, including melanoma, squamous cell carcinoma and basal cell carcinoma (see Jee, et al, *The Journal of Investigative Dermatology,* 119, 5, pp. 1121- 1127, 2002 and Ming, et al., *The Journal of Investigative Dermatology,* 129, pp. 2109-2112, 2009, copies enclosed), brain cancer (see Rong,et al., *PNAS,* 101, 52, pp. 18200-18205, December 28, 2004, copy enclosed) and ovarian cancer (see Wang,et al., *Oncogene,*24, 3574-3582, 2005, copy enclosed). These references evidence the dramatic generic role that phosphatidyl inositol 3-kinase plays in a variety of cancers and also evidences that inhibition of this enzyme is material to the treatment of these cancers.

33. Given the exceptional inhibitory activity solenopsin displays against

PAGE 24/45 * RCVD AT 12/6/2011 9:37:37 AM [Eastern Standard Time] * SVR:W-PTOFAX-001/26 * DNIS:2738300 * CSID:12038706002 * DURATION (mm-ss):09-35

phosphoinositol-3 kinase and the direct and indirect role that phosphoinositol-3 kinase plays in promoting angiogenesis, a critically important process in tumor/cancer growth and elaboration, as evidenced by the literature cited and enclosed, as well as the experimental test results which are described in paragraphs 29-31, above, it is my expectation that solenopsin will prove to be an effective agent against tumors and cancer by inhibiting angiogenesis in cancer tissue through inhibition of phosphoinositol-3 kinase. This expectation is corroborated by the favorable antiproliferative/anticancer activity exhibited by solenopsin in the cell-based assays which are described above and in attached Exhibits 1-4, as well as the literature cited which further evidences the broad generic role that phosphoinositol 3-kinase plays in cancer treatment. By virtue of the inhibitory activity of the compounds which are presently claimed in the pending method claims, it is my expectation that these compounds will prove to be effective anti-cancer agents against a broad range of tumors and cancer.

34. As a separate point, I understand that Examiner Zarek has objected to Figure 5 in the original application for the reasons which are cited in the July, 2011 office action on pages 6-7. Applicants submit that the entry for Solenopsin in figure 5, in particular for the entry at a concentration of 6 micrograms/mL, which presents data for an SVR inhibition assay conducted prior to the filing of the present application, is an artifact of the experimental conditions run at the time the figure was generated. The SVR assay, the results of which are presented in the original specification, may be viewed as a screen for preliminary results. The 6 microgram test result from the SVR assay of Figure 5 is an experimental artifact that may be attributed to a number of factors, including but not limited to aggregation effects, solubility, sample purity, etc. Occasionally, in the SVR assay, concentration effects have been observed. In such cases either the assay is repeated or other experiments are carried out. In this case, we have repeated this experiment several times and a more accurate experiment is presented in figure 2 of the previously submitted *Blood* 2007 paper, referenced above in paragraph 29. We have repeated the experiment several times with the same results which are presented in figure 2 of the *Blood 2007* paper. The combination of experiments described above unambiguously demonstrates that solenopsin A is inhibiting angiogenesis and by the akt

PAGE 25/45 * RCVD AT 12/6/2011 9:37:37 AM [Eastern Standard Time] * SVR:W-PTOFAX-001/26 * DNIS:2738300 * CSID:12038706002 * DURATION (mm-ss):09-35

pathway.

35. I further declare that all statements made herein of my own personal knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: _____ _____

Jack L. Abiser, MD, PhD

PAGE 26/45 * RCVD AT 12/6/2011 9:37:37 AM [Eastern Standard Time] * SVR:W-PTOFAX-001/26 * DNIS:2738300 * CSID:12038706002 * DURATION (mm-ss):09-35

pathway.

35. I further declare that all statements made herein of my own personal knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 12/5/11

Jack L. Abiser, MD, PhD

PAGE 27/45 * RCVD AT 12/6/2011 9:37:37 AM [Eastern Standard Time] * SVR:W-PTOFAX-001/26 * DNIS:2738300 * CSID:12038706002 * DURATION (mm-ss):09-35

EXHIBIT 1

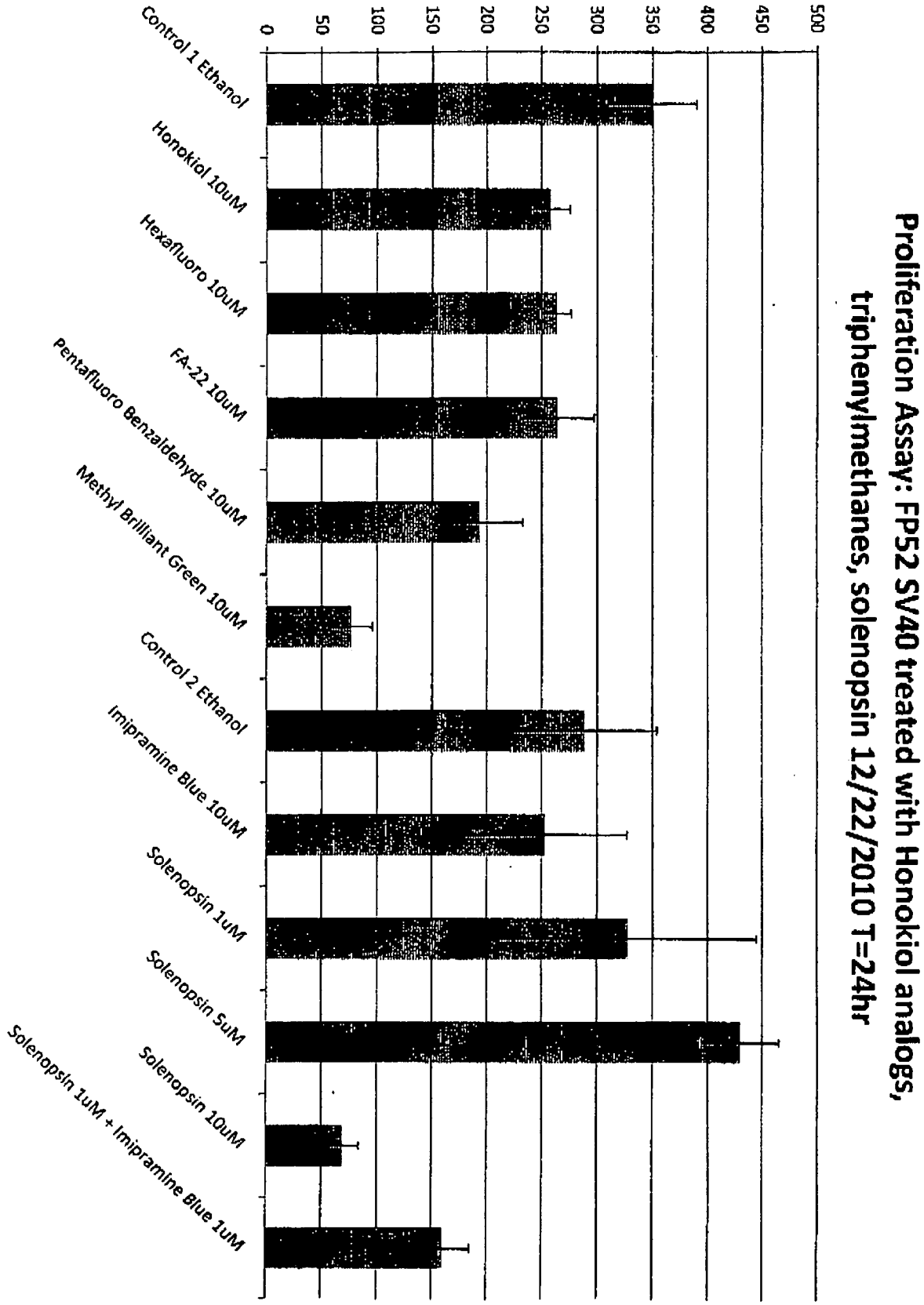

Proliferation Assay: FP52 SV40 treated with Honokiol analogs, triphenylmethanes, solenopsin 12/22/2010 T=24hr
0
50
100
150
200
250
300
350
400
450
500
Control 1 Ethanol
Honokiol 10uM
Hexafluoro 10uM
FA-22 10uM
Pentafluoro Benzaldehyde 10uM
Methyl Brilliant Green 10uM
Control 2 Ethanol
Imipramine Blue 10uM
Solenopsin 1uM
Solenopsin 5uM
Solenopsin 10uM
Solenopsin 1uM + Imipramine Blue 1uM

EXHIBIT 2

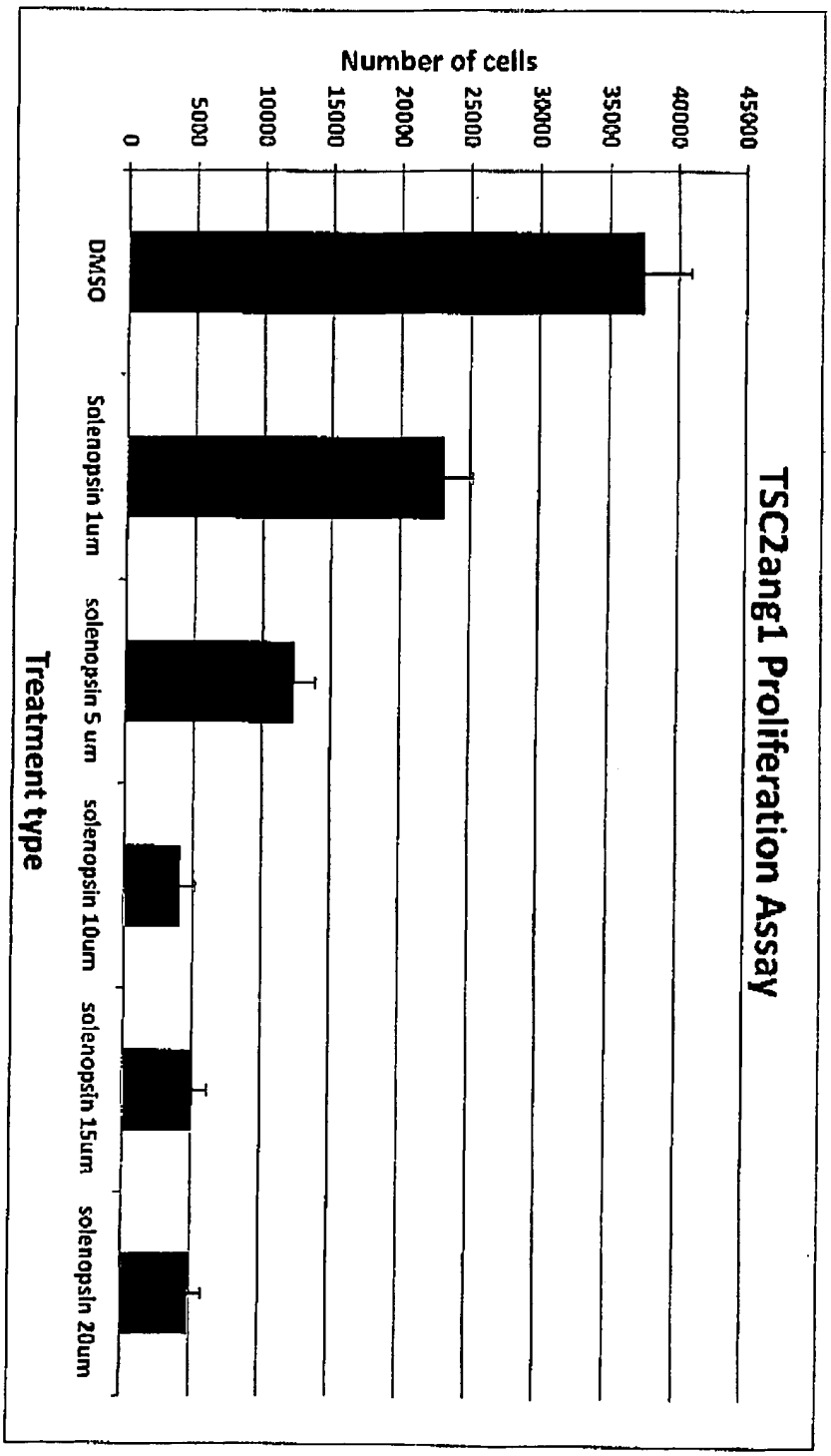

TSC2ang1 Proliferation Assay
Number of cells
0
5000
10000
15000
20000
25000
30000
35000
40000
45000
DMSO
Solenopsin 1um
solenopsin 5 um
solenopsin 10um
solenopsin 15um
solenopsin 20um
Treatment type

EXHIBIT 3

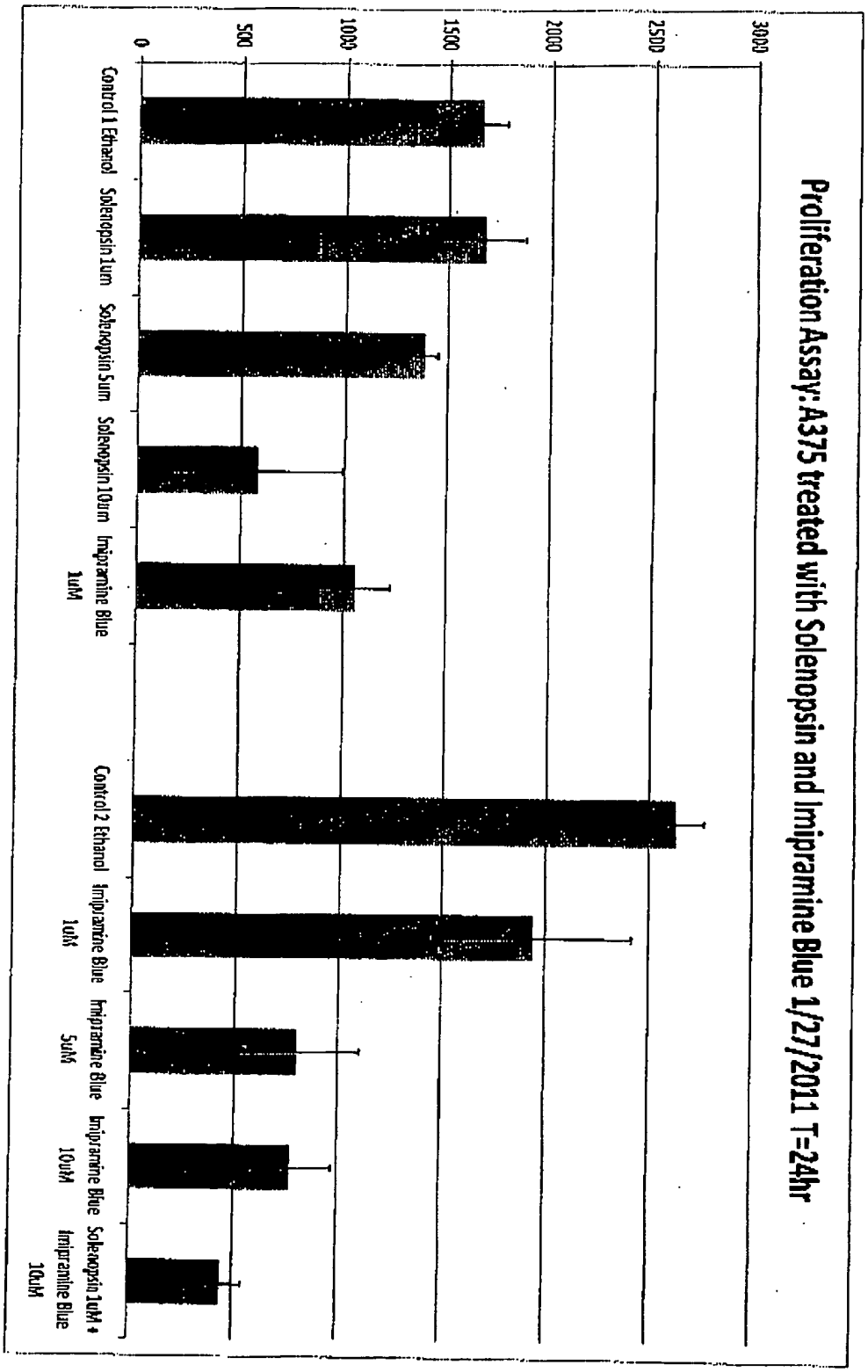
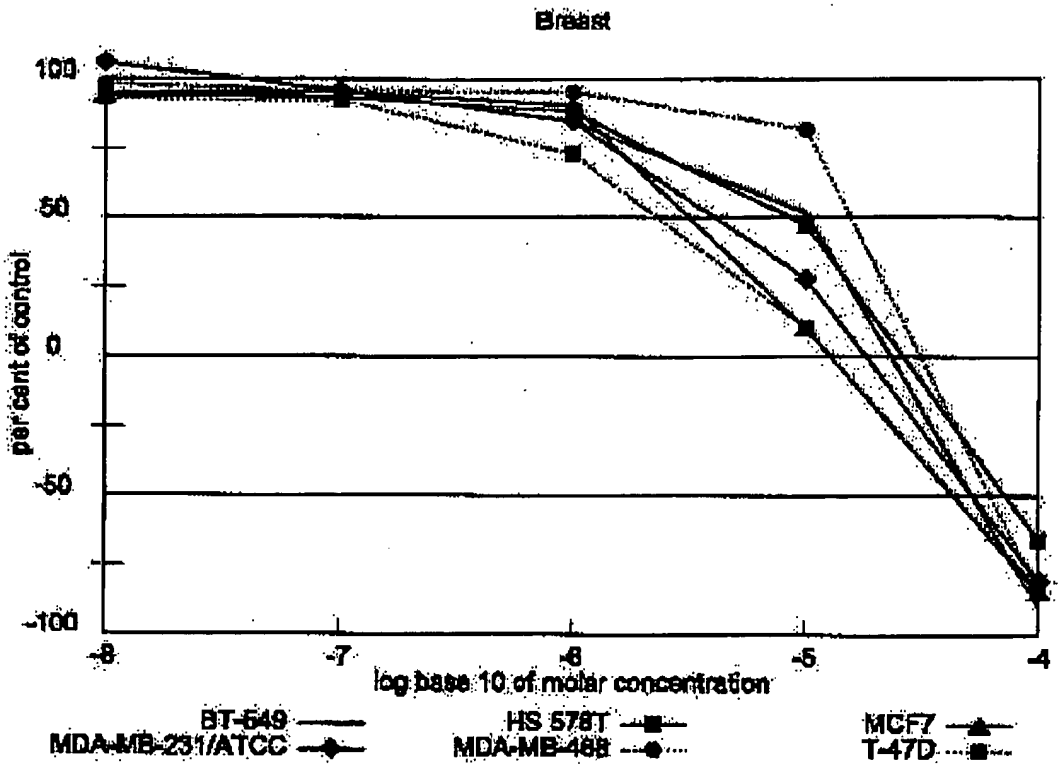
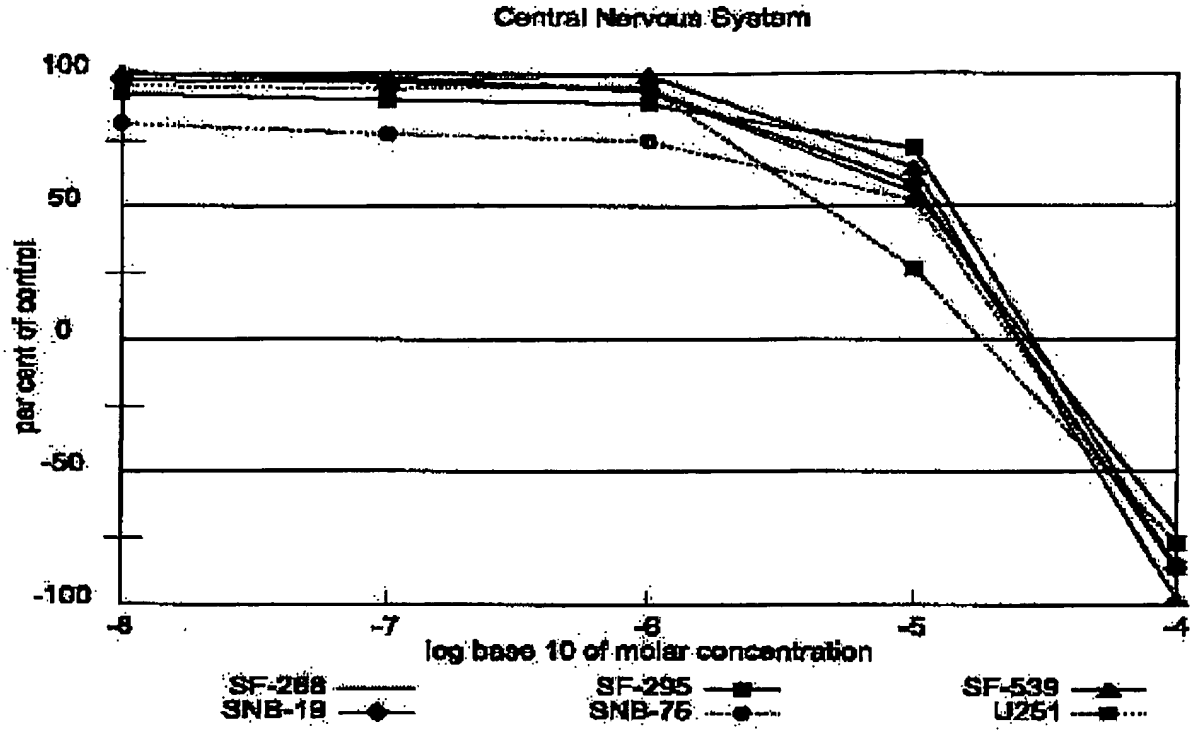
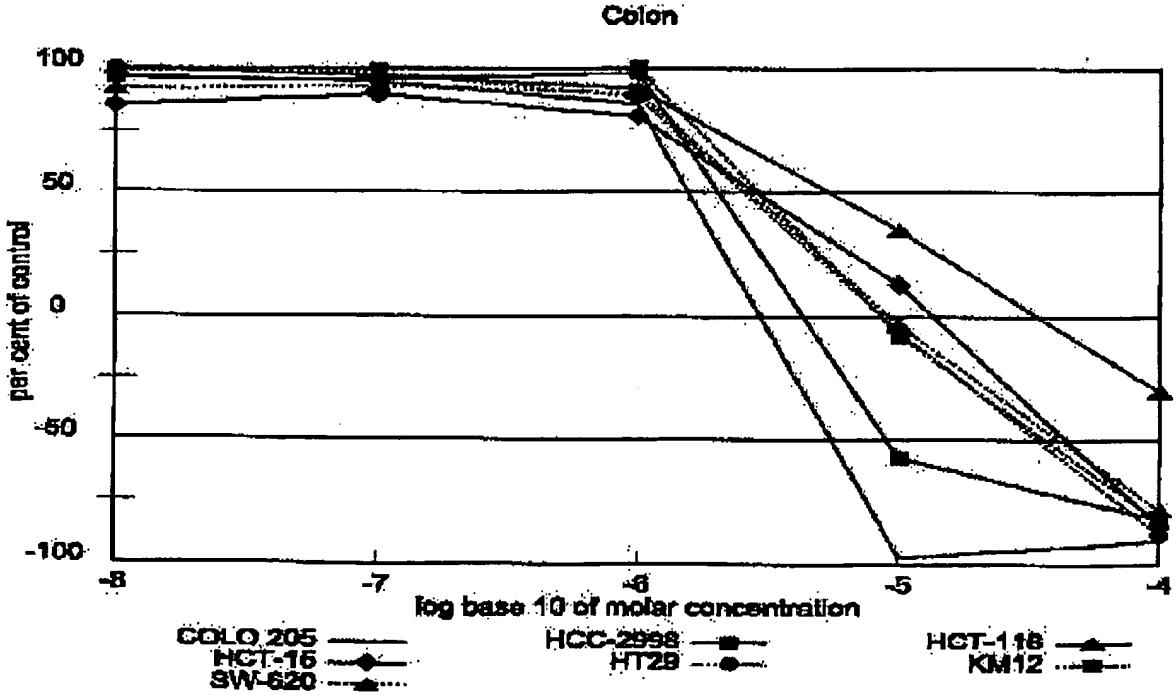

Proliferation Assay: A375 treated with Solenopsin and Imipramine Blue 1/27/2011 T=24hr
0
500
1000
1500
2000
2500
3000
Control 1 Ethanol
Solenopsin 1um
Solenopsin 5um
Solenopsin 10um
Imipramine Blue 1uM
Control 2 Ethanol
Imipramine Blue 1uM
Imipramine Blue 5uM
Imipramine Blue 10uM
Solenopsin 1uM + Imipramine Blue 10uM

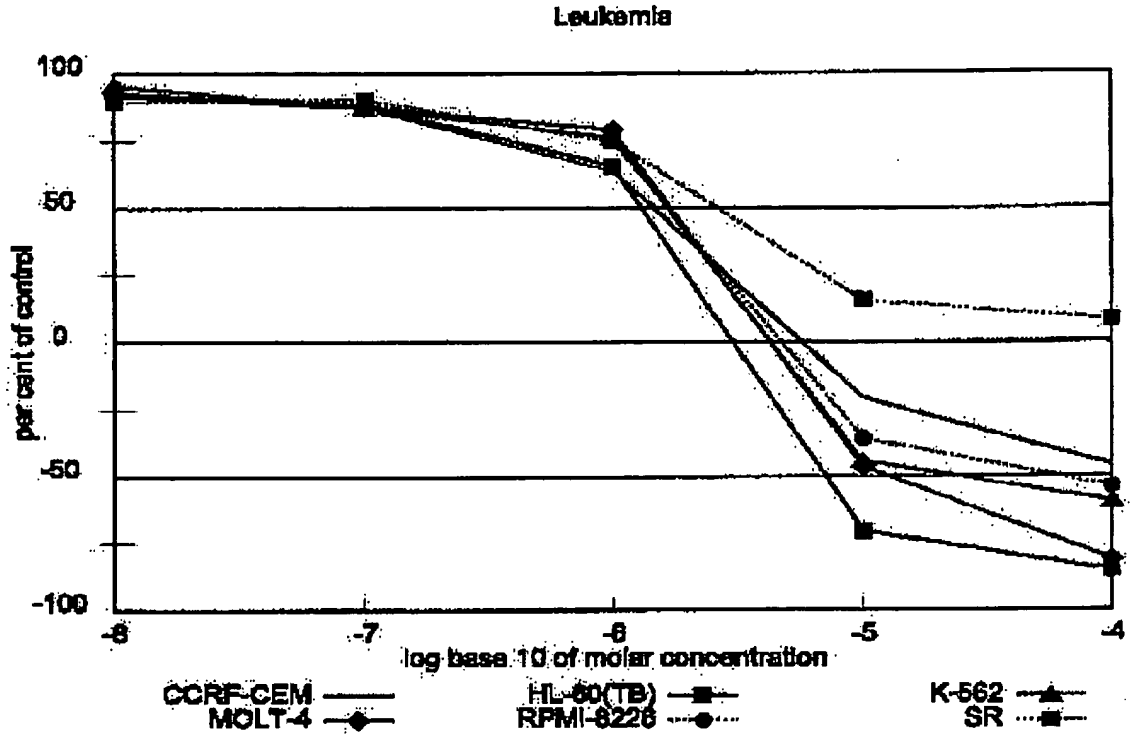
EXHIBIT 4

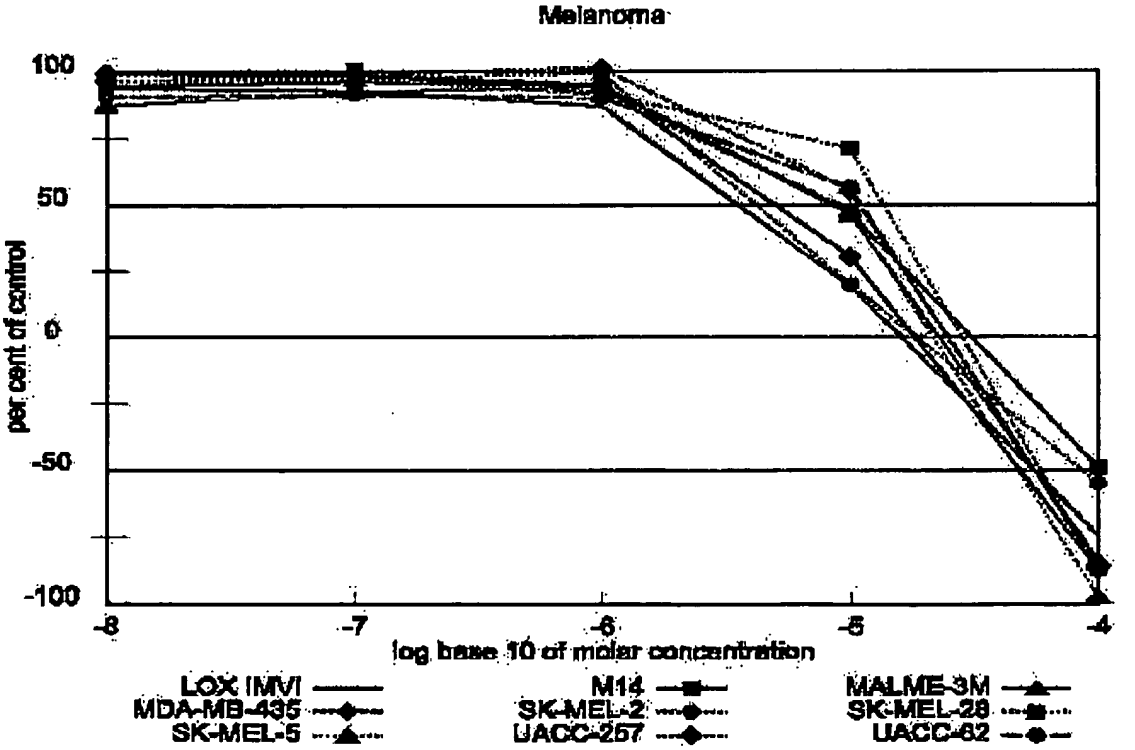
NATIONAL CANCER INSTITUTE DEVELOPMENTAL THERAPEUTICS PROGRAM
DOSE RESPONSE CURVES FOR NSC 166588
(SOLENOPSIN A)

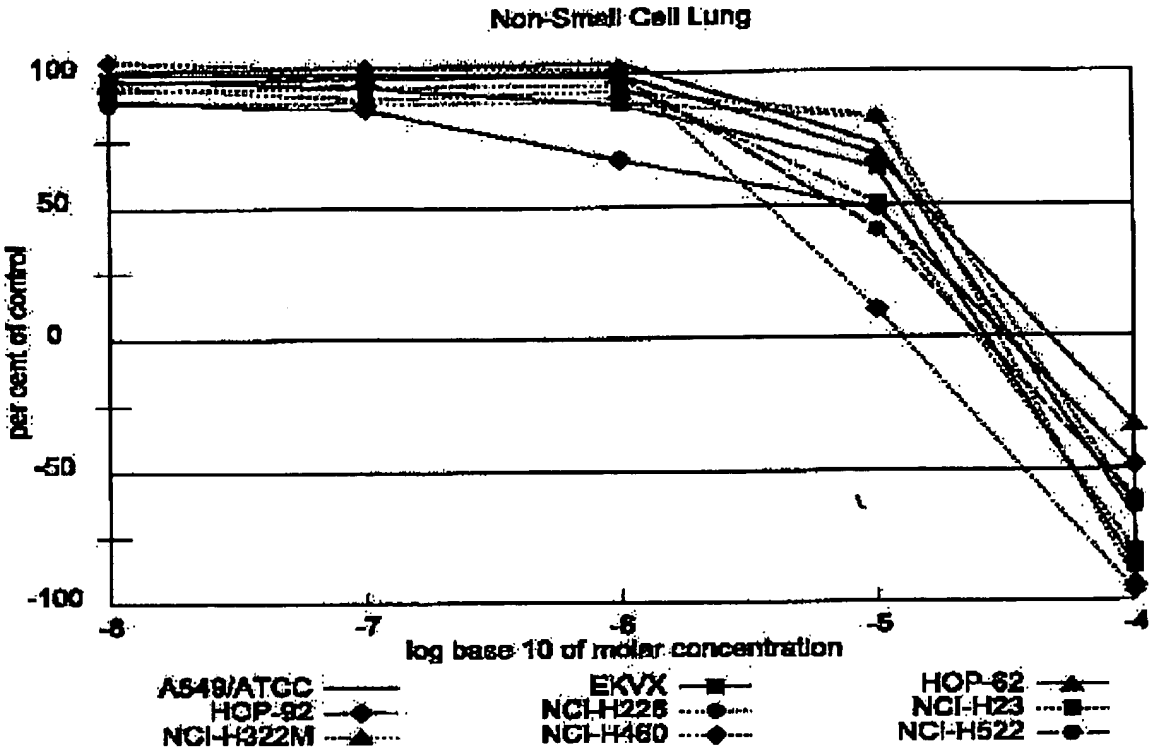


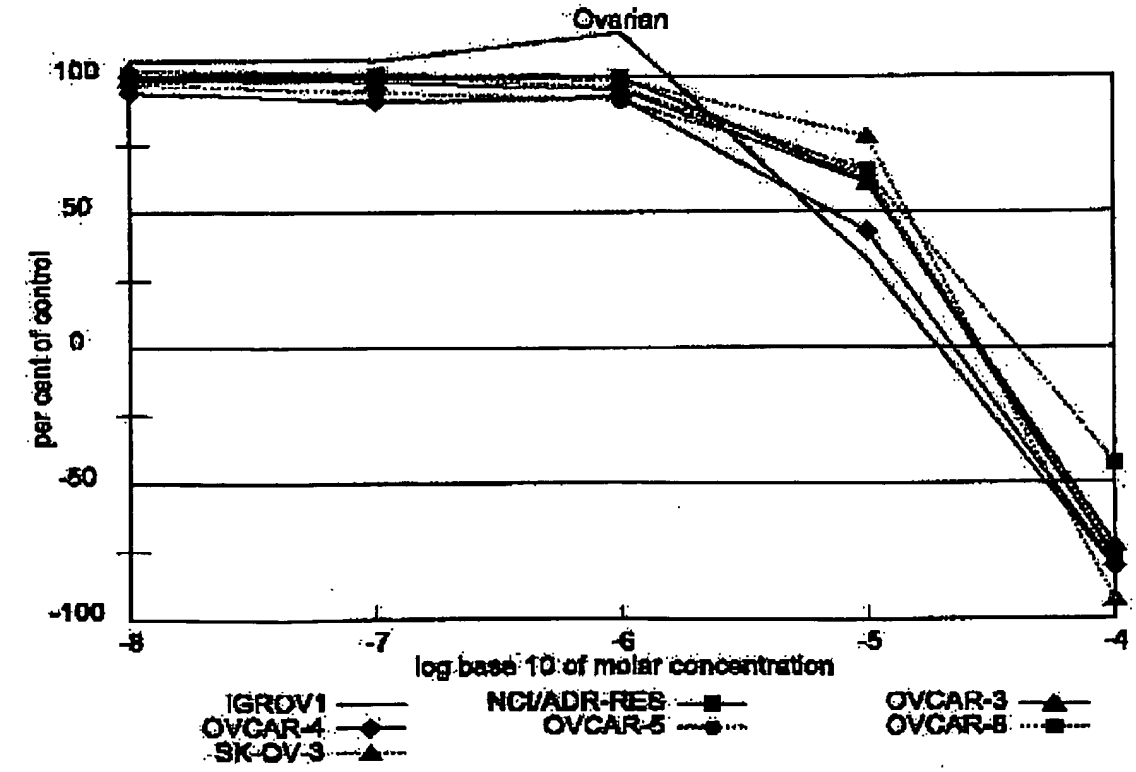

Central Nervous System
100
50
0
-50
-100
per cent of control
-8
-7
-6
-5
-4
log base 10 of molar concentration
SF-268
SF-295
SF-539
SNB-19
SNB-75
U251

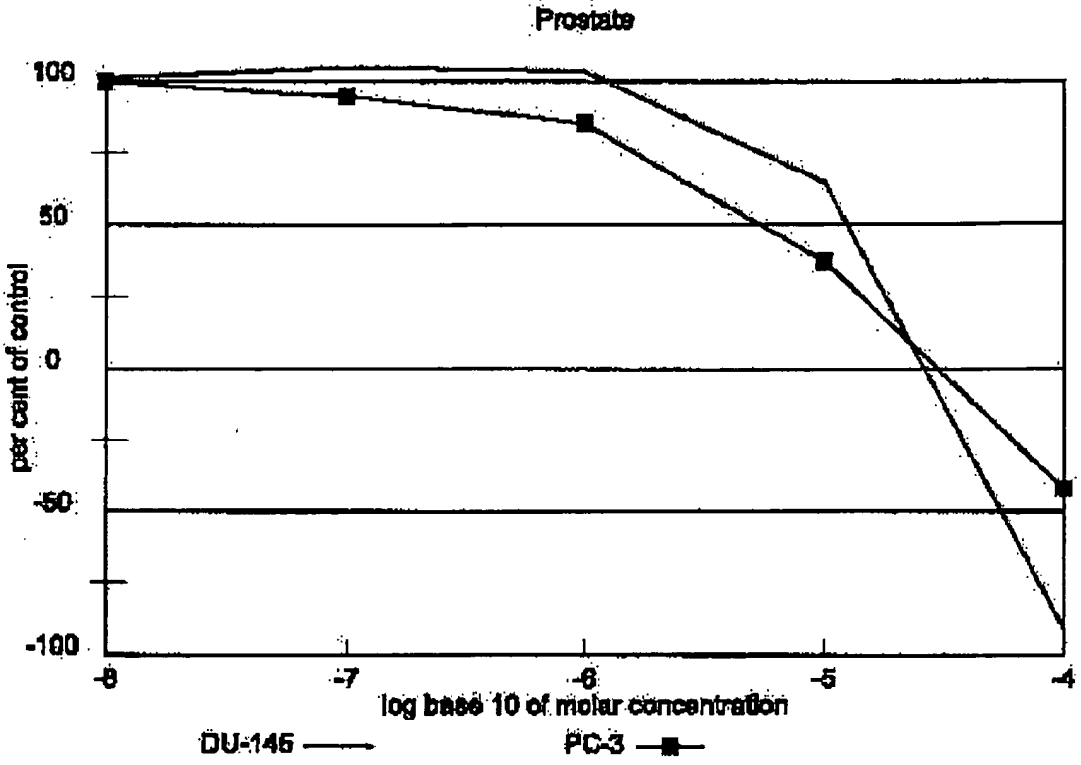

Colon
100
50
0
-50
-100
per cent of control
-8
-7
-6
-5
-4
log base 10 of molar concentration
COLO 205
HCC-2998
HCT-116
HCT-15
HT29
KM12
SW-620

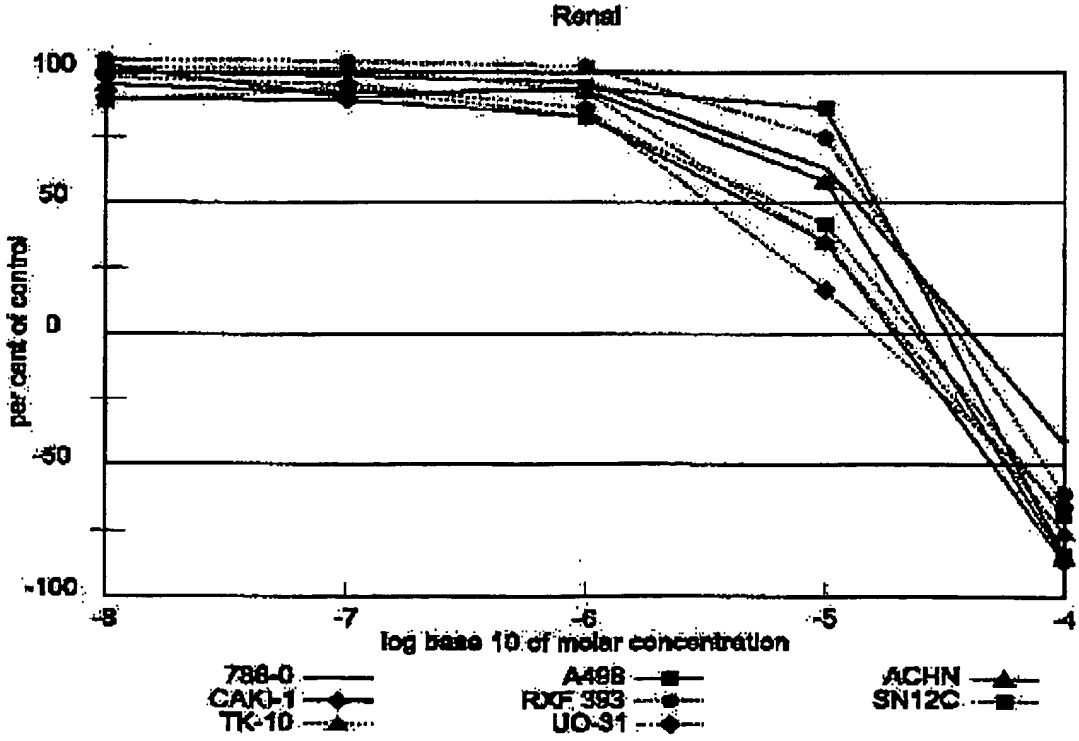

Leukemia
100
50
0
-50
-100
per cent of control
-8
-7
-6
-5
-4
log base 10 of molar concentration
CCRF-CEM
MOLT-4
HL-60(TB)
RPMI-8226
K-562
SR


Melanoma
100
50
0
-50
-100
per cent of control
-8
-7
-6
-5
-4
log base 10 of molar concentration
LOX IMVI
MDA-MB-435
SK-MEL-5
M14
SK-MEL-2
UACC-257
MALME-3M
SK-MEL-28
UACC-62


Non-Small Cell Lung
100
50
0
-50
-100
per cent of control
-8
-7
-6
-5
-4
log base 10 of molar concentration
A549/ATCC
EKVX
HOP-62
HOP-92
NCI-H226
NCI-H23
NCI-H322M
NCI-H460
NCI-H522


Ovarian
100
50
0
-50
-100
per cent of control
-8
-7
-6
-5
-4
log base 10 of molar concentration
IGROV1
NCI/ADR-RES
OVCAR-3
OVCAR-4
OVCAR-5
OVCAR-8
SK-OV-3


Prostate
100
50
0
-50
-100
per cent of control
-8
-7
-6
-5
-4
log base 10 of molar concentration
DU-145
PC-3


Renal
100
50
0
-50
-100
per cent of control
-8
-7
-6
-5
-4
log base 10 of molar concentration
786-0
CAKI-1
TK-10
A498
RXF 393
UO-31
ACHN
SN12C

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 28, Issue of July 11, pp. 25490–25498, 2003
Printed in U.S.A.
© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.

Inhibition of NF-κB Sensitizes A431 Cells to Epidermal Growth Factor-induced Apoptosis, whereas Its Activation by Ectopic Expression of RelA Confers Resistance*

Received for publication, February 19, 2003, and in revised form, April 16, 2003
Published, JBC Papers in Press, April 24, 2003, DOI 10.1074/jbc.M301790200

Ruby John Anto, Manickam Venkatramani, and Devarajan Karunagaran§
From the Division of Cancer Biology, Rajiv Gandhi Center for Biotechnology, Thiruvananthapuram, Kerala-695014, India

Epidermal growth factor (EGF) is a well known mitogen, but it paradoxically induces apoptosis in cells that overexpress its receptor. We demonstrate for the first time that the EGF-induced apoptosis is accelerated if NF-κB is inactivated. To inactivate NF-κB, human epidermoid carcinoma cells (A431) that overexpress EGF receptor were stably transfected with an IκB-α double mutant construct. Under the NF-κB-inactivated condition, A431 cells were more sensitive to EGF with decreased cell viability and increased externalization of phosphatidylserine on the cell surface, DNA fragmentation, and activation of caspases (3 and 8 but not 9), typical features of apoptosis. These results were further supported by the potentiation of the growth inhibitory effects of EGF by chemical inhibitors of NF-κB (curcumin and sodium salicylate) and the protective role of RelA evidenced by the resistance of A431-RelA cells (stably transfected with RelA) to EGF-induced apoptosis. EGF treatment or ectopic expression of RelA in A431 cells induced DNA binding activity of NF-κB (p50 and RelA) and the expression of c-IAP1, a downstream target of NF-κB. A431-RelA cells exhibited spontaneous phosphorylation of Akt (a downstream target of phosphatidylinositol 3-kinase and regulator of NF-κB) and EGF treatment stimulated it further. Blocking this basal Akt phosphorylation with LY294002, an inhibitor of phosphatidylinositol 3-kinase, did not affect their viability but blocking of EGF-induced phosphorylation of Akt sensitized the otherwise resistant A431-RelA cells to EGF-mediated growth inhibition. Our results favor an anti-apoptotic role for NF-κB in the regulation of EGF-induced apoptosis.

Epidermal growth factor (EGF)[1] is a polypeptide (6-kDa) that belongs to the EGF family of ligands (heparin binding EGF, transforming growth factor-α, amphiregulin, β-cellulin, epiregulin, and neuregulins) binding to specific cell surface receptors (1, 2). Upon ligand binding, the epidermal growth factor receptor (EGFR) dimerizes, autophosphorylates itself, and recruits a cascade of signaling molecules before transmitting potent mitogenic signals in many cellular systems (1, 3). EGFR is overexpressed in a number of human malignancies including cancers of the lung, head and neck, brain, bladder, and breast (4). Furthermore, increased EGFR expression correlates with a poorer clinical outcome for patients with breast and ovarian cancers (5, 6). Whereas EGF is a potent mitogen, it paradoxically induces apoptosis in cells that overexpress EGFR such as A431 (7). Experimentally increasing the level of EGFR expression in epithelial, mesenchymal, or glial cells also leads to ligand-dependent apoptosis (8). Another ligand of the EGF family, heregulin (also known as neuregulin), is known to induce apoptosis in cells that overexpress ErbB2, the second member of the EGFR family (9). In addition, epiregulin also inhibited cell growth in EGFR-overexpressing cells (10). Induction of amphiregulin mRNA was observed in EGF-induced apoptosis (11) and interaction of EGF with pro-heparin-binding EGF leads to growth inhibition and apoptosis (12). Growth factors other than EGF such as platelet-derived growth factor and hepatocyte growth factor can also trigger cell cycle arrest and death in a variety of cells (13, 14) and in addition, EGF enhanced the apoptotic effect of platelet-derived growth factor (14). An EGF-related protein, Cripto-1, promotes apoptosis in HC-11 mouse mammary epithelial cells (15). Anoikis, activation of EGFR tyrosine kinase, Ras-MAP kinase signaling, and the elevation of Stat1 and p21 levels have been advocated as mechanisms driving EGF-induced apoptosis (11, 16–18) but, the actual mechanism appears to be more elusive and complicated.

Apoptosis or programmed cell death is a physiological process characterized by distinct morphological and biochemical features that include membrane blebbing, chromatin condensation, cytoplasmic shrinking, DNA fragmentation, and activation of different caspases (19). Typically two different pathways, extrinsic receptor-mediated and intrinsic mitochondria-mediated, leading to apoptosis have been identified (20, 21). Mostly cytokines of the tumor necrosis factor (TNF) superfamily induce apoptosis by interaction of the ligand with its death receptor, which sequentially recruits TNF receptor-associated death domain, Fas-associated death domain, caspase 8, and caspase 3, the last then cleaves various substrates leading to apoptosis. In contrast, the mitochondria-mediated pathway involves the release of cytochrome *c* from the mitochondria, and cytochrome *c* together with Apaf1 activates caspase 9, and the latter then activates caspase 3, resulting in apoptosis (20, 21). Tumor cells often evade apoptosis by expressing several anti-apoptotic proteins such as Bcl-2, down-regulation and mutation of pro-apoptotic genes and alterations of p53, PI-3K/Akt, or NF-κB

* This work was supported in part by grants from the Department of Science and Technology, Government of India and the Science, Technology and Environment committee, Government of Kerala, India (to D. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a Senior Research Fellowship from the Council of Scientific and Industrial Research, India.

§ To whom correspondence should be addressed. Tel.: 91-471-2347975; Fax: 91-471-2348096; E-mail: dkarunagaran@hotmail.com.

[1] The abbreviations used are: EGF, epidermal growth factor; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor receptor; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; IκB, inhibitor κB; IKK, IκB kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; PARP, poly-(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PI, propidium iodide; PS, phosphatidylserine; TNF, tumor necrosis factor; IAP, inhibitor of apoptosis protein; PI-3K, phosphatidylinositol 3-kinase; AFC, 7-amino-4-trifluoromethyl coumarin; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

This paper is available on line at http://www.jbc.org

Downloaded from www.jbc.org at Univ of California - Irvine, on October 14, 2011

PAGE 40/45 * RCVD AT 12/6/2011 9:37:37 AM [Eastern Standard Time] * SVR:W-PTOFAX-001/26 * DNIS:2738300 * CSID:12038706002 * DURATION (mm-ss):09-35

pathways that give them survival advantage and thereby resist therapy-induced apoptosis (20).

NF-κB is a family of transcription factors activated by a diverse number of stimuli including EGF, cytokines, such as TNF-α and interleukin-1, UV irradiation, and lipopolysaccharides (22). EGF has been reported to activate NF-κB in smooth muscle cells, fibroblasts, and in several EGFR-overexpressing cell lines (23–25). Binding of IκB to NF-κB masks nuclear localization signals and prevents its translocation to the nucleus (26). Stimulation of cells with a diverse array of stimuli results in phosphorylation of IκB-α on serines 32 and 36 at its NH_2-terminal. This leads to the ubiquitination and degradation of IκB-α, allowing NF-κB to translocate to the nucleus and activate transcription (22, 26). Inhibition of NF-κB activity potentiates cell killing of human breast cancer and fibrosarcoma cell lines by TNF-α, ionizing radiation, and daunorubicin (27–29). NF-κB inhibition sensitized tumors in mice to chemotherapeutic compound CPT-11-mediated cell killing (30). NF-κB directly causes increased expression of proteins that contribute to the survival of tumor cells such as inhibitors of apoptotic proteins (IAPs) (31, 32). Results from our laboratory have shown earlier that ectopic expression of the RelA subunit of NF-κB into murine fibrosarcoma cells protects them from curcumin-induced apoptosis (33).

To understand whether NF-κB plays any role in EGF-induced apoptosis, we used A431 cells that overexpress EGFR and stably transfected them with a mutant IκB (known to inactivate NF-κB) or RelA (known to activate NF-κB). Using several parameters to assess apoptosis such as viability, externalization of phosphatidylserine (PS) on the cell surface, DNA fragmentation, and activation of caspases we report that A431 cells are more sensitive to EGF-induced apoptosis under NF-κB-inactivated conditions whereas its activation confers resistance.

EXPERIMENTAL PROCEDURES

Reagents, Chemicals, and Antibodies—EGF (isolated from male mouse submaxillary glands), Dulbecco's minimum essential medium, and fetal bovine serum were procured from Invitrogen. Curcumin, sodium salicylate, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate mixture, and a mouse monoclonal antibody to β-actin (A-5441) were purchased from Sigma. Fluorimetric substrates for caspase 3 (Ac-DEVD-AFC number 264157) and caspase 9 (Ac-LEHD-AFC number 218765) were obtained from Calbiochem. Rabbit polyclonal antibodies to p50 (sc-7178), RelA (sc-109), hemagglutinin (HA) (sc-7392), IκB-α (sc-271), and c-JAP1 (sc-7943) were procured from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to caspase 8 (1C12), rabbit polyclonal PARP antibody (number 9542), and phospho-Akt pathway sampler kit (number 9916 containing antibodies to Akt and phospho-Akt, and LY294002) were purchased from Cell Signaling Technology (Beverly, MA), and the mouse monoclonal EGFR antibody (clone 111) raised against the extracellular domain of EGFR was a gift from Dr. Yosef Yarden, Weizmann Institute of Science, Israel.

Cell Lines and Culture—Human epidermoid carcinoma cell line A431 was obtained from the National Center for Cell Science, Pune, India. The cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (Invitrogen). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Transient and Stable Transfections—A431-IκB-α cells were transiently transfected with *relA* in pMT2T vector (33, 34) using the calcium-phosphate transfection kit (Invitrogen) according to the manufacturer's protocol. Stable transfections in A431 cells with *relA* in pMT2T vector (co-transfected with pcDNA3) or the empty vector pcDNA3 or pcDNA3-IκB-α were carried out by the LipofectAMINE method (35). For the preparation of liposome solution, 20 μmol/ml of stock (prepared by mixing 6.6 μmol of dimethyl dioctadecyl ammonium bromide and 13.4 μmol of dioleyl-α-phosphatidylethanolamine in 1 ml of ethanol) was diluted into 1 nmol/μl in water. For transfection, the cells were seeded to attain 70% confluence in 35-mm Petri dishes. For each dish, 2 μg of DNA and 24 μl of liposome solution were mixed in 500 μl of Dulbecco's minimum essential medium free from serum and antibiotics, vortexed, and incubated at room temperature for 30 min. The liposome solution (1 ml) was layered over the cells previously rinsed with serum-free medium and left for 4 h in a CO_2 incubator and then the medium was replenished with 20% fetal bovine serum and reverted back to 10% fetal bovine serum after 24 h. After 72 h, cells were grown in selection medium (400 μg/ml G418) and clones formed were picked up and maintained separately with 100 μg/ml G418.

Western Blotting—Cells were lysed in whole cell lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, 0.1% Triton X 100, 1 mM DTT, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 4 mM sodium orthovanadate). Equal amounts of total protein for each sample were separated by SDS-PAGE and electrotransferred onto nitrocellulose filters, probed with the primary antibodies and appropriate peroxidase-conjugated secondary antibodies, and visualized with the enhanced chemiluminescence (ECL) method as per the manufacturer's protocol (Amersham Biosciences). For some experiments, alkaline phosphatase-conjugated secondary antibodies from Sigma were used and the bands were detected using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate.

Electrophoretic Mobility Shift Assay (EMSA)—Cells were washed with cold PBS and suspended in 160 μl of lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml benzamidine). The cells were allowed to swell for 30 min, after which 4.5 μl of 10% Nonidet P-40 was added, vortexed, and centrifuged, and the pellet was suspended in 25 μl of nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml benzamidine). The nuclear extract (8 μg of protein) collected after 30 min by centrifugation was used to perform EMSA by incubating it with 16 fmol of ^{32}P-end labeled 45-mer double stranded NF-κB oligonucleotide from the human immunodeficiency virus-1 long terminal repeat (5′-TTGTTACAAGGCACTTTCCGCTGG-GGACTTTCCAGGGAGGCGTGG-3′) in the presence of 1 μg/ml poly(dI-dC) in a binding buffer (25 mM HEPES (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, and 5% glycerol) for 30 min at 37 °C and the DNA-protein complex was resolved using a 6.6% native polyacrylamide gel. The gels were dried and the radioactive bands were visualized by phosphorimaging (Bio-Rad Personal FX).

MTT Assay—For MTT assay, 25 μl of MTT solution (5 mg/ml in PBS) was added to cells (untreated and treated) cultured in 96-well plates. Cells were incubated for 2 h and 0.1 ml of the extraction buffer (20% sodium dodecyl sulfate in 50% dimethyl formamide) was added after removal of MTT with a PBS wash. After an overnight incubation at 37 °C, the optical densities at 570 nm were measured using a plate reader (Bio-Rad), with the extraction buffer as a blank. The relative cell viability in percentage was calculated as (A_{570} of treated samples/A_{570} of untreated samples) × 100.

[^{3}H]Thymidine Incorporation—Cells grown in 96-well plates were treated with or without the indicated concentrations of EGF and at the end of 18 h, [^{3}H]thymidine was added to each well (0.5 μCi/well) and the incubation was continued for a total period of 24 h. The culture medium was then removed, washed twice with PBS, and the proteins were precipitated with 5% trichloroacetic acid. The supernatant was removed and after washing with ethanol, the cells were solubilized with 0.2 N NaOH, and the radioactivity was counted using a liquid scintillation counter.

Annexin-PI Staining—The cells (10^6 cells/well) were seeded in 48-well plates and treated with or without EGF for 16 h. Then the cells were washed with PBS and treated with 1× assay buffer, annexin-fluorescein isothiocyanate and propidium iodide (PI) as per the protocol described in the annexin V apoptosis detection kit (sc-4252 AK) from Santa Cruz Biotechnology. After 10–20 min, the wells were washed with PBS and greenish apoptotic cells were viewed using a Nikon fluorescent microscope and photographed.

Comet Assay—Comet assay was carried out essentially as described (36). Briefly, the cells (treated with or without EGF) were pelleted and resuspended in 0.5% low melting point agarose at 37 °C and layered on a frosted microscope slide previously coated with a thin layer of 0.5% normal melting agarose and kept for 5 min at 4 °C. After solidification, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.5, 1% Triton X-100, and 10% Me_2SO) for 1 h at 4 °C. The slides were then electrophoresed for 20 to 30 min at 25 V. The slides after electrophoresis were washed with 0.4 M Tris (pH 7.5) and stained with ethidium bromide (1 μg/ml) and observed under a Nikon fluorescent microscope.

Downloaded from www.jbc.org at Univ of California - Irvine, on October 14, 2011

Assays of Caspase 3 and Caspase 9—The enzymatic activities of caspase 3 or caspase 9 were assayed spectrofluorimetrically (37). Briefly, the whole cell lysate was incubated with 50 μM fluorimetric substrates of caspase 3 (Ac-DEVD-AFC) or caspase 9 (Ac-LEHD-AFC) in a total volume of 500 μl of reaction buffer (50 mM HEPES-KOH, pH 7.0, 10% glycerol, 0.1% CHAPS, 2 mM EDTA, 2 mM DTT) at 37 °C for 1 h. The released AFC was quantitated using a spectrofluorimeter (PerkinElmer LS-50 B) with the excitation and emission wavelengths of 380 and 460 nm, respectively. Values of relative fluorescence units released per mg of protein were calculated.

RESULTS

EGF Induces NF-κB DNA Binding Activity in A431-Neo but Not A431-IκB-α Cells—The human epidermoid carcinoma cell line, A431, overexpresses EGFRs (about 2 × 10^6 EGFRs/cell) and has been extensively used as a model system to study the effects of EGF on cell proliferation (7, 38–40). To study the role of NF-κB in EGF-induced apoptosis, we used A431 cells and stably transfected them with either the empty vector (pcDNA3) or pcDNA3-IκB-α, a double mutant construct in which both the serines (32 and 36) at the amino-terminal of IκB-α are mutated to alanine. Because the double mutant form of IκB-α lacks the crucial serine residues that need to be phosphorylated by NF-κB activators, it is popularly employed to strongly inhibit NF-κB (28). As the construct, pcDNA3-IκB-α, contains the hemagglutinin tag (HA tag), Western blotting of HA protein was used to ascertain the presence of the mutant IκB protein. As expected, all six clones of A431-IκB-α cells (selected by G418) showed the presence of HA protein whereas the A431-Neo cells transfected with control vector (pcDNA3) did not show its expression (Fig. 1*A*). Further experiments with IκB-transfected cells were done using only clone 6 of A431-IκB-α cells (showing very high HA expression) unless stated otherwise. Western blotting with a polyclonal IκB-α antibody confirmed that clone 6 had indeed a higher level of IκB-α expression compared with that in the parental as well as A431-Neo cells (Fig. 1*B*). For the Western blots, β-actin was used as a control and the results confirm equal loading of the samples (Fig. 1, *A* and *B*). To see whether transfection procedures affected the level of EGFR, Western blotting was carried out in parental, A431-Neo, and A431-IκB-α cells and the results confirmed that EGFR levels remained unaffected in these cells (Fig. 1*C*). Because the very purpose of stable transfection was to inactivate NF-κB, it was logical to check whether EGF could stimulate the NF-κB DNA binding activity in A431-Neo and A431-IκB-α cells (clone 6) by EMSA. Whereas 50 ng/ml EGF enhanced the NF-κB DNA binding activity in A431-Neo cells, even 100 ng/ml EGF could not induce it in A431-IκB-α cells and in the absence of EGF there were no active DNA-binding complexes of NF-κB in both the cells (Fig. 1*D*). To confirm whether the active complex contains the classical NF-κB partners, p50 and RelA, the nuclear extracts prepared from A431-Neo cells stimulated with 50 ng/ml EGF were incubated with antibodies to RelA or p50 and then EMSA was carried out. As shown in Fig. 1*E*, both antibodies shifted the active NF-κB complex (supershift) whereas incubation with excess of an unlabeled oligonucleotide containing the NF-κB binding site removed the active complex. Fig. 1*E* also shows that transient transfection of A431-IκB-α cells with *relA* restored the NF-κB active complex, and *relA* transfection was used by us earlier to constitutively activate NF-κB in L-929 cells (33). These results confirm that the A431-IκB-α cells express the mutant form of IκB-α that inactivated NF-κB and hence, EGF could not stimulate NF-κB DNA binding activity in these cells. The results also indicate that EGF can induce the NF-κB DNA binding activity in A431-Neo cells and the active NF-κB complex contains the heterodimers, p50 and RelA. In addition, the results show that RelA, being one of the heterodimeric partners of active NF-κB, favors the formation of active NF-κB DNA binding complexes and has the potential to reverse the NF-κB-inactivating effect of IκB.

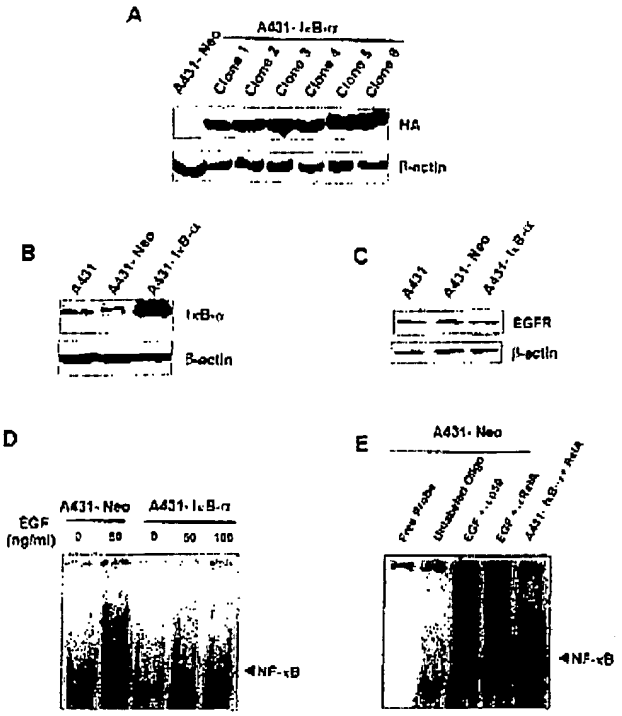


FIG. 1. **Western blotting for HA and IκB-α, and assessment of NF-κB DNA binding activity in transfected and untransfected A431 cells.** *A*, A431 cells were transfected with pcDNA3 vector or the pcDNA3-IκB-α construct using LipofectAMINE and the G418-resistant clones were selected as described under "Experimental Procedures." Cell lysates (40 μg of protein) from the vector-transfected A431-Neo cells and the different clones of A431-IκB-α cells were subjected to SDS-PAGE and immunoblotted with HA antibody or β-actin (control) by ECL as described under "Experimental Procedures." *B*, cell lysates from A431, A431-Neo, and the clone-6 of A431-IκB-α cells were subjected to Western blotting with an antibody to IκB-α or β-actin (control) as described above. *C*, similarly, the cell lysate from A431, A431-Neo, or A431-IκB-α cells was subjected to Western blotting with an antibody to EGFR or β-actin (control). *D*, A431-Neo cells or A431-IκB-α cells grown in 35-mm Petri dishes (1 × 10^6 cells/dish) were treated with EGF at the indicated concentrations at 37 °C for 1 h. Nuclear extracts were prepared and EMSA was done as described under "Experimental Procedures." *E*, A431-Neo cells were treated with 50 ng/ml EGF as described above and EMSA was done as before. The nuclear extracts from EGF-stimulated cells were also incubated with either RelA or p50 antibody or with 10 times excess of unlabeled oligo. One of the lanes had the nuclear extract prepared from A431-IκB-α cells transiently transfected with *relA* as described under "Experimental Procedures" and another lane contained the labeled oligo (free probe) without the addition of nuclear extract. All the experiments above were done at least two times with similar results. The *arrowheads* shown in *panels D* and *E* indicate the positions of the active DNA-binding complexes of NF-κB.

A431-IκB-α Cells Are More Sensitive to EGF-induced Cytotoxicity and DNA Fragmentation Than A431-Neo Cells—To study the effects of EGF on cell growth under conditions of NF-κB inactivation, A431-Neo and A431-IκB-α cells were exposed to various concentrations of EGF and the cell viability in percentage over untreated control was determined after 72 h by MTT assay. The MTT assay is a convenient screening assay for the measurement of cell death while it does not discriminate between apoptosis and necrosis. EGF treatment at 0.01 and 0.1 ng/ml had a stimulatory effect on A431-Neo cells with cell viabilities of 130 and 110%, respectively, if the viability of untreated control was taken as 100% at the end of 72 h (Fig. 2*A*). In contrast, EGF inhibited the A431-IκB-α cells with only 81 and 67% of cells being alive for the concentrations of 0.01

Downloaded from www.jbc.org at Univ of California - Irvine, on October 14, 2011

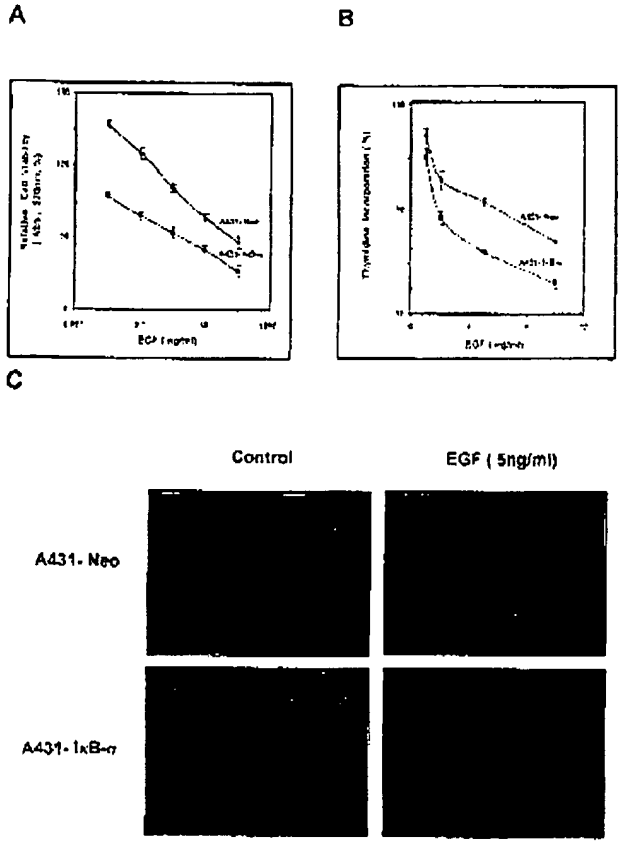


FIG. 2. **Inhibition of growth and DNA synthesis and induction of DNA fragmentation in A431-Neo and A431-IκB-α cells by EGF.** *A*, cells grown in 96-well plates (5×10^3 cells/well) were incubated for 72 h either with medium or different concentrations of EGF as indicated and cell viability was assessed by the MTT assay as described under "Experimental Procedures." *B*, cells grown in 96-well plates (5×10^3 cells/well) were incubated with different concentrations of EGF for 24 h, and then the thymidine incorporation as a measure of DNA synthesis was determined as described under "Experimental Procedures" and the results are expressed as percentage over the control (untreated cells). The above experiments were repeated two times with similar results and all determinations were made in triplicate and the *error bars* show the standard deviations. *C*, cells (1×10^6) were seeded in 35-mm Petri dishes and incubated with 5 ng/ml EGF for 24 h. After incubation, cells were trypsinized, pelleted, and the comet assay was done as described under "Experimental Procedures." The experiment was repeated once more with similar results.

and 0.1 ng/ml, respectively, compared with the control (Fig. 2*A*). As can be seen in Fig. 2*A*, EGF at a concentration of 1 ng/ml and above was inhibitory to both A431-Neo and A431-IκB-α cells. Thus, even at concentrations that were stimulatory to A431-Neo cells, EGF inhibited the growth of A431-IκB-α cells (Fig. 2*A*) suggesting that A431-IκB-α cells are much more sensitive to EGF-induced cytotoxicity and these results were also confirmed in 2 other clones of A431-IκB-α (data not shown). We also examined the effect of EGF on DNA synthesis by the method of thymidine incorporation after exposing the cells to different concentrations of EGF (1–10 ng/ml) found to be inhibitory to both A431-Neo and A431-IκB-α cells by the MTT assay. EGF inhibited DNA synthesis in both A431-Neo and A431-IκB-α cells in a dose-dependent manner and again the A431-IκB-α cells were more sensitive to EGF-induced inhibition of DNA synthesis (Fig. 2*B*). DNA fragmentation is another hallmark of apoptosis and to detect this, we have used the single cell gel electrophoresis (Comet assay), a sensitive technique that allows detection of DNA strand breaks. DNA strand breaks create fragments that when embedded in agarose gels migrate in an electric field. Cells with damaged DNA when stained with ethidium bromide appear like a comet and the length of the comet tail represents the extent of DNA damage. Fig. 2*C* clearly indicates that well formed comets are more in number in A431-IκB-α than A431-Neo cells when induced with 5 ng/ml EGF while the untreated cells did not exhibit the comet morphology in both the cells. These results suggest that A431-IκB-α cells with inactivated NF-κB are more susceptible to cell death induced by EGF compared with A431-Neo cells.

Relatively More A431-IκB-α Cells Undergo EGF-induced Externalization of Phosphatidylserine—To assess whether the cell death induced by EGF involves typical changes encountered during apoptosis, we first looked for changes in PS on the cell membrane. Under defined salt and calcium concentrations, annexin V is predisposed to bind PS that is externalized to the cell surface in the very early stages of apoptosis (41, 42). Hence, apoptotic cells were detected using annexin V labeled with fluorescein isothiocyanate and photographed with a camera-attached fluorescent microscope. Addition of PI helps to distinguish the early apoptotic cells from late apoptotic or necrotic cells because PI cannot enter the cells in the early stages of apoptosis when the membrane integrity is intact (41, 42). In A431-IκB-α cells 37 and 65% were annexin positive after treatment with 5 and 10 ng/ml EGF, respectively, whereas only 9 and 23% of the A431-Neo cells showed annexin positivity (greenish yellow) for the same corresponding EGF concentrations (Fig. 3). Untreated A431-Neo and A431-IκB-α cells either did not show annexin positivity or had a very minimum number of positively stained cells (Fig. 3). However, a small percentage of A431-Neo and A431-IκB-α cells also showed typical PI staining (yellowish red) suggesting the appearance of late apoptotic or necrotic cells with 10 ng/ml EGF treatment (Fig. 3). These results indicate that in comparison with A431-Neo cells, a relatively large number of A431-IκB-α cells exhibit externalization of PS, a typical feature of apoptosis upon treatment with EGF.

A431-IκB-α Cells Are More Sensitive to EGF-induced Apoptosis That Involves Cleavage of PARP, Activation of Caspases 3 and 8, but Not Caspase 9—In many systems caspase 8 and caspase 9 act as initiator caspases and caspase 3, the effector, signals for the final execution of the cells. Pro-caspase 8 was cleaved into its active fragments (p43/41 and p18) by 10 ng/ml EGF in A431-Neo cells while even 5 ng/ml EGF could easily do it in A431-IκB-α cells as visualized by Western blotting and the untreated cells did not show any of the cleavage products (Fig. 4*A*). Caspase 3 activity was determined spectrofluorimetrically using a substrate, Ac-DEVD-AFC, an acetylated synthetic tetrapeptide corresponding to the upstream amino acid sequence of the caspase 3 cleavage site in PARP, and the fluorphor AFC (7-amino-4-trifluromethyl coumarin). Whereas EGF-induced caspase 3 activity was 2.1-fold more than the untreated control in A431-IκB-α cells, it was only 1.2-fold more than the control in A431-Neo cells (Fig. 4*B*). Caspase 9 activity was also determined fluorimetrically and EGF treatment could not induce caspase 9 activity in A431-IκB-α and A431-Neo cells, whereas curcumin used as a positive control activated caspase 9 in both cells (Fig. 4*C*). We also examined the cleavage of a well characterized caspase 3 substrate, PARP, from its 116-kDa intact form into the 89-kDa fragment by Western blotting. When A431-IκB-α cells were treated with 5 or 10 ng/ml EGF, the 116-kDa form of PARP decreased and the 89-kDa form increased indicating that the full-length PARP was converted to an apoptotic fragment while the untreated cells showed only the uncleaved fragment (Fig. 4*D*). In A431-Neo cells, both control and 5 ng/ml EGF-treated cells did not exhibit the cleaved

Downloaded from www.jbc.org at Univ of California - Irvine, on October 14, 2011

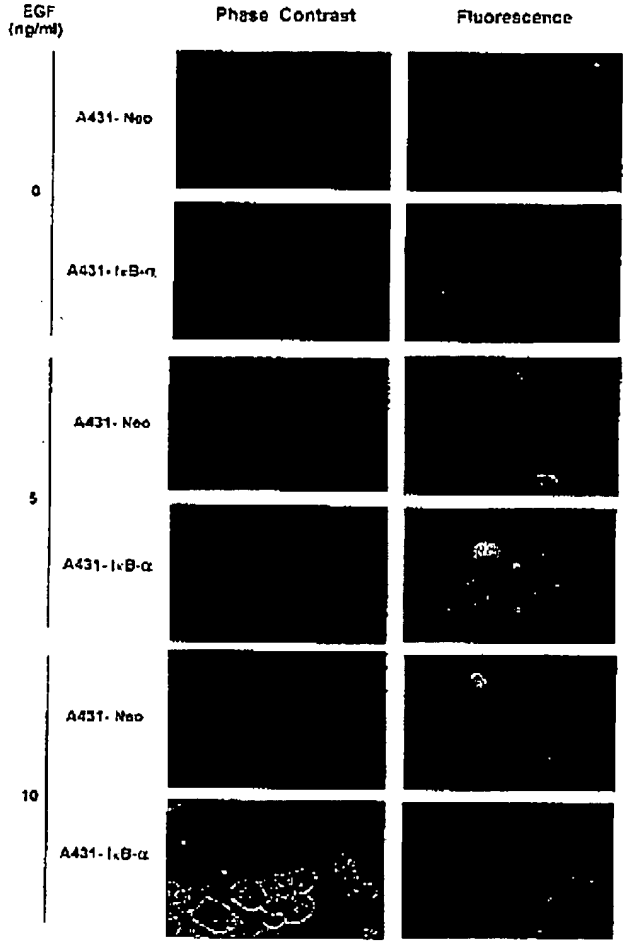


FIG. 3. **EGF-induced changes in annexin-PI staining.** Cells were incubated with or without the indicated concentrations of EGF for 16 h, and stained for annexin-PI positivity (not shown in color) as described under "Experimental Procedures." The same results were confirmed in another independent experiment.

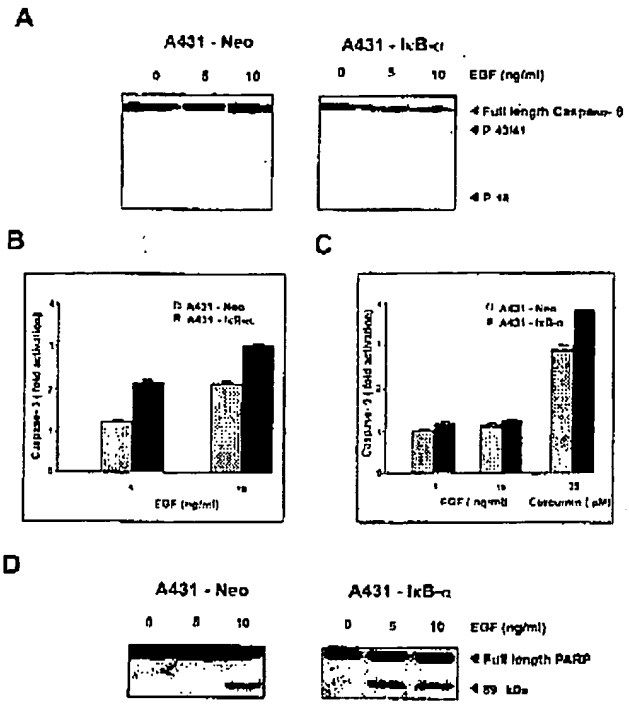


FIG. 4. **Effects of EGF on the activities of caspases 8, 3, and 9, and PARP cleavage.** *A*, cells (1×10^6) were seeded in 35-mm Petri dishes and treated with or without EGF for 24 h. To detect the active caspase 8 fragments, the cell lysates were resolved on 12% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, probed with caspase 8 antibody (1:3000), and detected by the alkaline phosphatase method using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate. *B*, cells (1×10^6) were seeded in 35-mm Petri dishes and treated with or without EGF for 24 h. Fifty micrograms of total protein was incubated with 50 μM caspase 3 fluorimetric substrate in a total volume of 500 μl of the reaction buffer and the fluophor released was quantitated spectrofluorimetrically as described under "Experimental Procedures." *C*, cells (1×10^6) were seeded in 35-mm Petri dishes and treated with EGF or curcumin along with untreated control for 24 h. Fifty micrograms of total protein was incubated with 50 μM caspase 9 fluorimetric substrate and the activity was quantitated spectrofluorimetrically as above. The reproducibility of these experiments was ascertained by repeating them at least two times. *D*, cells (1×10^6) were seeded in 35-mm Petri dishes and treated with or without EGF for 24 h. To detect the cleavage of PARP, whole cell lysate (40 μg) was resolved on a 7.5% polyacrylamide gel, electrotransferred, probed with PARP antibody (1:3000), and detected by ECL reagent as described earlier. Similar results were obtained when the experiment was repeated.

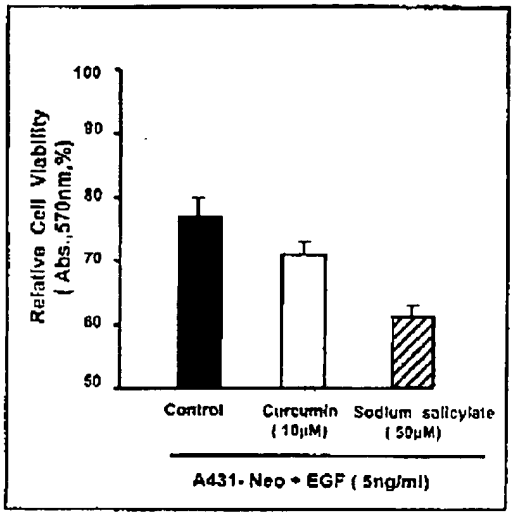
product and even 10 ng/ml EGF could induce only a slight PARP cleavage (Fig. 4*D*). The above results confirm that the A431-IκB-α cells are more sensitive to EGF-induced apoptosis than A431-Neo cells by showing increased PARP cleavage and higher activation of caspases 3 and 8 but not 9 suggesting the operation of a caspase 8-mediated extrinsic pathway.

Chemical Inhibitors of NF-κB Enhance the Susceptibility of A431-Neo Cells whereas Activation of NFκB by RelA Reverses the Susceptibility of A431-IκB-α Cells to EGF-induced Cytotoxicity—To know whether chemical inhibitors of NF-κB would also enhance EGF-induced apoptosis, the A431-Neo cells were treated with known inhibitors of NF-κB such as sodium salicylate (50 μM) or curcumin (10 μM) for 2 h prior to EGF treatment. When compared with untreated cells, 77% A431-Neo cells were alive in wells treated with 5 ng/ml EGF for 24 h (Fig. 5*A*). If the viability of A431-Neo cells treated with 10 μM curcumin alone for 24 h was taken as 100, treatment with EGF after pretreatment with curcumin reduced it to 71% (Fig. 5*A*). Similarly, if 50 μM sodium salicylate pretreatment is compared with or without EGF the viability of A431-Neo cells was only 61% (Fig. 5*A*). If IκB-mediated inhibition of NF-κB can positively regulate EGF-induced apoptosis, then this effect is expected to be reversed by NF-κB. As expected, the transient transfection of *relA* into A431-IκB-α cells partly reversed the cytotoxic effect of EGF as measured by an MTT assay (Fig. 5*B*).

The higher expression of RelA in the transfected cells was confirmed by Western blotting and to ensure that proteins were loaded equally, β-actin controls were used (Fig. 5*B*, *inset*). These results suggest that similar to IκB, NF-κB inhibitors also have the potential to enhance EGF-induced cell death and NF-κB has a protective role suggested by the higher level of resistance of A431-IκB-α cells transiently transfected with *relA* to EGF-induced cytotoxicity.

RelA Protects A431 Cells from EGF-induced Apoptosis—Because RelA reversed the effect of IκB-α it became relevant to know whether, on its own, it can protect the parental A431 cells from EGF-induced apoptosis. To this end, we transfected the A431 cells stably with *relA* and confirmed the higher expression of RelA in A431-RelA cells (clone 1 and clone 2) compared with A431 and A431-Neo cells by Western blotting (Fig. 6*A*). When A431-Neo and A431-RelA cells (clone 1) were compared for their relative viability in the presence of varying concentrations of EGF by the MTT assay, A431-RelA cells were notably more resistant to EGF (Fig. 6*B*) and these results were also confirmed using clone 2 (data not shown)

Downloaded from www.jbc.org at Univ of California - Irvine, on October 14, 2011



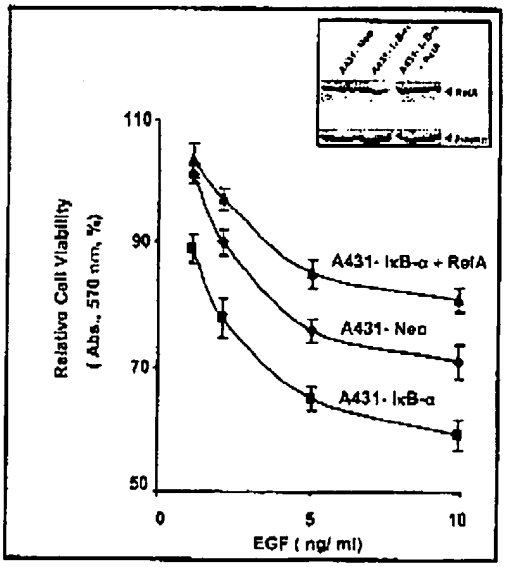


FIG. 5. **Influence of chemical inhibitors of NF-κB and *relA* transfection on EGF-mediated growth inhibition of A431-Neo and A431-IκB-α cells, respectively.** *A*, A431-Neo cells (5×10^3/well) were seeded into 96-well plates and pretreated with 10 μM curcumin, 50 μM sodium salicylate, or medium alone for 2 h. The cells were then treated with or without 5 ng/ml EGF for 24 h and the MTT assay was done as described earlier. Triplicate samples were used and the *error bars* indicate the standard deviations and the results were confirmed in another independent experiment. *B*, A431-IκB-α cells were transiently transfected with *relA* as described under Fig. 1*E*. A431-Neo and A431-IκB-α and the transfected cells (A431-IκB-α+RelA) were seeded into 96-well plates (6×10^3/well) and treated with the indicated concentrations of EGF for 24 h and the MTT assay was done as described earlier. Triplicate samples were used and the *error bars* indicate the standard deviations. The results were confirmed in another independent experiment. The *inset* shows Western blots of RelA and β-actin control in A431-Neo, A431-IκBα, and the transfected cells (*A431-IκB-α+RelA*).

but further experiments were done using clone 1 of A431-RelA cells. Similarly EGF-mediated inhibition of thymidine incorporation was relatively more in A431-Neo than A431-RelA cells confirming the protective role of RelA against

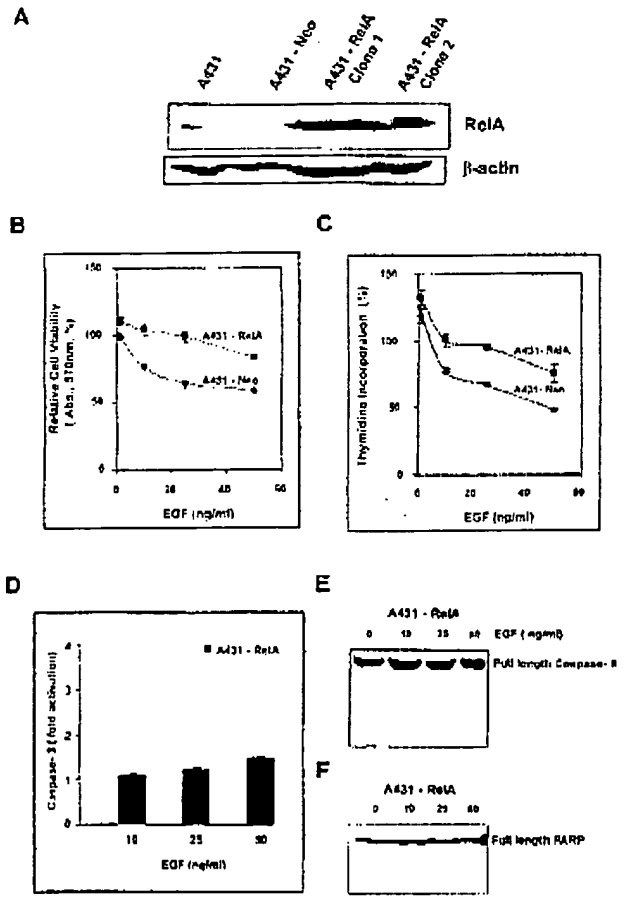


FIG. 6. **Western blotting for RelA and EGF-mediated changes in growth, DNA synthesis, caspase activation, and PARP cleavage.** *A*, A431 cells were cotransfected with pcDNA3 vector and *relA* PMT2T construct using LipofectAMINE and the G418-resistant clones were selected as described under "Experimental Procedures." Cell lysates (60 μg of protein) from the vector-transfected A431-Neo cells and different clones of A431-RelA cells were subjected to SDS-PAGE and immunoblotted with RelA or β-actin (control) antibody by the alkaline phosphatase method as described under "Experimental Procedures." *B*, cells were treated with or without the indicated concentrations of EGF for 24 h and the MTT assay was done under the conditions described for Fig. 2. Triplicate samples were used and the *error bars* indicate the standard deviations. The results were confirmed in another independent experiment. *C*, cells were treated with or without the indicated concentrations of EGF for 24 h and thymidine incorporation assays were done under the conditions described for Fig. 2. Triplicate samples were used and the *error bars* indicate the standard deviations. The results were confirmed in another independent experiment. *D*, A431-RelA cells were treated with or without the indicated concentrations of EGF for 24 h and caspase 8 activity was determined as described for Fig. 4. *E*, cells were treated with or without the indicated concentrations of EGF for 24 h and caspase 3 activity was determined as described for Fig. 4. *F*, cells were treated with or without the indicated concentrations of EGF for 24 h and PARP cleavage was determined as described for Fig. 4.

EGF-mediated cell death (Fig. 6*C*). In addition, varying EGF concentrations even up to 50 ng/ml could not induce caspase 8 (Fig. 6*D*) or caspase 3 (Fig. 6*E*) activities or PARP cleavage (Fig. 6*F*) in A431-RelA cells.

EGF Up-regulates the Expression of c-IAP1 in A431-Neo Cells and Its Basal Expression Is Higher in A431-RelA Cells—As IAP is considered to be one of the survival proteins induced by NF-κB, it was of interest to study the effect of EGF and RelA on IAP expression. Expression of c-IAP1 was observed by Western blotting in A431-Neo cells stimulated with 10 ng/ml EGF for

Downloaded from www.jbc.org at Univ of California - Irvine, on October 14, 2011